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# Systemic Hematologic Effects of PEG-rHuMGDF-Induced Megakaryocyte Hyperplasia in Mice

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PEG-rHuMGDF injected daily in normal mice causes a rapid dose-dependent increase in megakaryocytes and platelets. At the same time that platelet numbers are increased, the mean platelet volume (MPV) and platelet distribution width (PDW) can be either decreased, normal, or increased depending on the dose and time after administration. Thus, PEG-rHuMGDF at a low dose causes decreases in MPV and PDW, MGDF at an intermediate dose causes an initial increase followed by a decrease in MPV and PDW, and PEG-rHuMGDF at higher doses causes an increase in MPV and PDW followed by a gradual normalization of these platelet indices. In addition to the expected thrombocytosis after 7 to 10 days of daily injection of high doses of PEG-rHuMGDF, a transient decrease in peripheral red blood cell numbers and hemoglobin is noted accompanied in the bone marrow by megakaryocytic hyperplasia, myeloid hyperplasia, ery-

throid and lymphoid hypoplasia, and deposition of a fine network of reticulin fibers. Splenomegaly, an increase in splenic megakaryocytes, and extramedullary hematopoiesis accompany the hematologic changes in the peripheral blood and marrow to complete a spectrum of pathologic features similar to those reported in patients with myelofibrosis and megakaryocyte hyperplasia. However, all the PEG-rHuMGDF-initiated hematopathology including the increase in marrow reticulin is completely and rapidly reversible upon the cessation of administration of PEG-rHuMGDF. Thus, transient hyperplastic proliferation of megakaryocytes does not cause irreversible tissue injury. Furthermore, PEG-rHuMGDF completely ameliorates carboplatin-induced thrombocytopenia at a low-dose that does not cause the hematopathology associated with myelofibrosis.

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**T**HROMBOPOIETIN (TPO) was identified in aplastic canine plasma as a growth factor for megakaryocytes.<sup>1</sup> Dog, mouse, and human TPO cDNA clones were subsequently isolated and the recombinant human TPO protein produced in mammalian cells has been shown to be biologically active in mice.<sup>2</sup> TPO binds to c-Mpl affinity columns and the in vitro biological effects of TPO are inhibited by soluble c-Mpl.<sup>2</sup> The activity of the c-Mpl ligand has been independently identified or cloned by several groups of investigators<sup>2-7</sup> who have begun to document the in vitro and in vivo thrombopoietic effects of this novel protein. Megakaryocyte growth and development factor (PEG-rHuMGDF), a truncated pegylated molecule related to human TPO, is likewise potent in vitro, in normal mice, and in experimental animal models. MGDF, even lacking pegylation, has been shown to ameliorate the decrease in platelets caused by carboplatin or by a combination of carboplatin and radiation.<sup>8-9</sup>

The purpose of the present study is to report that: (1) Systemic administration of truncated pegylated human recombinant *Escherichia coli*-derived c-mpl ligand (PEG-rHuMGDF) causes the dose-response and time-dependent appearance of marrow megakaryocytes and circulating platelets in mice, (2) PEG-rHuMGDF causes increases and decreases in mean platelet volume (MPV) and platelet distribution width (PDW) depending on the dose and time after administration, (3) PEG-rHuMGDF administration at high

doses is associated with development of a spectrum of pathologic features widely reported in patients with myelofibrosis accompanied by increased numbers of marrow megakaryocytes (anemia, splenomegaly, extramedullary hematopoiesis, and marrow pathology including myeloid hyperplasia, ery-

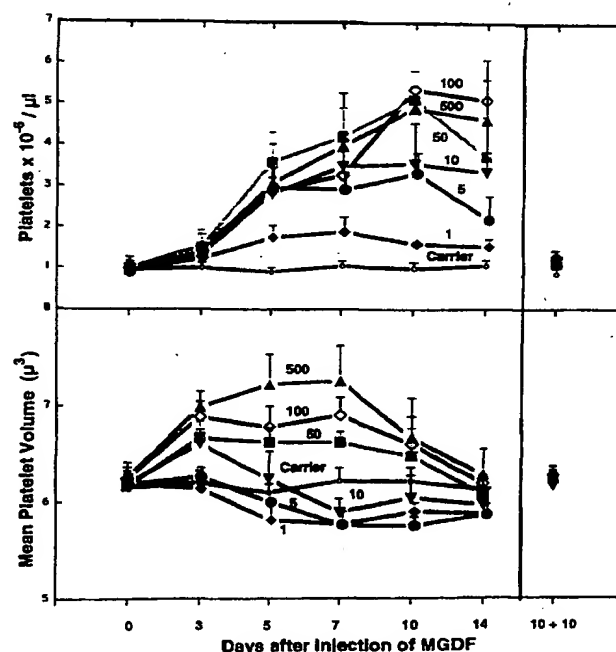


Fig 1. PEG-rHuMGDF injected once daily causes a statistically significant increase in the number of circulating platelets in a dose-response dependent fashion ( $n = 30$  mice in each dosage group at each time point; results expressed as mean  $\pm$  STD). PEG-rHuMGDF at a low dose causes a uniphasic decrease in MPV, at an intermediate dose causes an initial increase followed by a decrease in MPV, and at higher doses causes an increase in MPV followed by a gradual return to normal size. Injection of PEG-rHuMGDF for 10 days followed by cessation of treatment for 10 days results in a return of platelet number and MPV to control values ( $n = 5$  mice in each dosage group).

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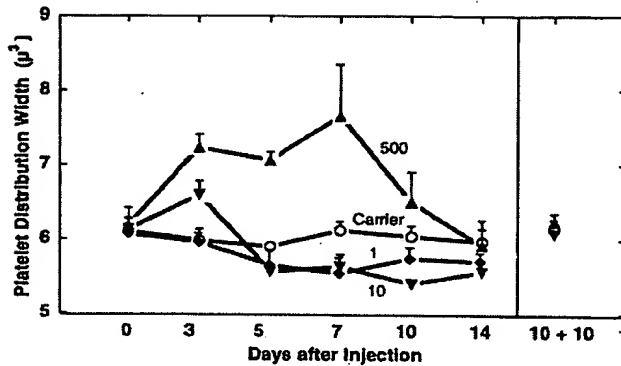


Fig 2. PEG-rHuMGDF injected once daily causes statistically significant changes in PDW that mirror those in MPV ( $n = 30$  mice in each dosage group at each time point; results expressed as mean  $\pm$  STD). PEG-rHuMGDF at  $1 \mu\text{g/kg}$  causes a decrease in PDW, at  $10 \mu\text{g/kg}$  causes an initial increase followed by a decrease in PDW, and at  $500 \mu\text{g/kg}$  causes an increase in PDW followed by a gradual normalization. Injection of PEG-rHuMGDF for 10 days followed by cessation of treatment for 10 days results in a return of PDW to control values ( $n = 5$  mice in each dosage group).

throid and lymphoid hypoplasia, and deposition of a fine network of reticulin fibers), (4) PEG-rHuMGDF-initiated hematopathology including the increase in marrow reticulin is completely and rapidly reversible upon the cessation of administration of PEG-rHuMGDF, and (5) PEG-rHuMGDF abrogates carboplatin-induced thrombocytopenia at a low-dose that does not cause the hematopathology associated with myelofibrosis.

#### MATERIALS AND METHODS

Human recombinant MGDF (1.1 to 2.1 mg/mL and  $<2.5$  EU/mL by limulus lysate assay) was expressed in *E. coli* and derivitized with

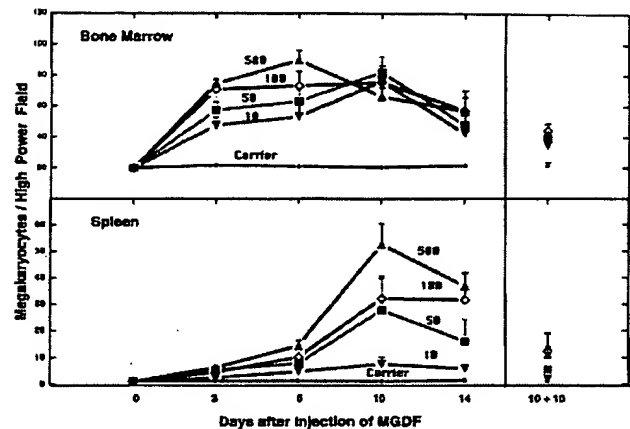


Fig 4. PEG-rHuMGDF causes a statistically significant increase in marrow megakaryocytes per high-power field to near peak numbers at day 3 ( $n = 10$  mice in each dosage group at each time point; 10 separate  $400\times$  high-power fields/femur examined and the results expressed as mean  $\pm$  STD per one high-power field). Splenic megakaryocytes do not increase until later. Injection of MGDF for 10 days followed by cessation of treatment for 10 days results in a continued decline of megakaryocyte numbers towards control values ( $n = 5$  mice in each dosage group).

polyethyleneglycol. PEG-rHuMGDF was injected once daily intraperitoneally (IP) in a carrier solution of 1% normal mouse serum dissolved in sterile saline. Balb/c mice ( $n = 490$ ) weighing approximately 20 g and 8 weeks of age (Charles River Labs, Wilmington, MA) and with access to food and water ad libitum were used for all in vivo experiments. Peripheral blood was collected by tail bleeding mice held in a restrainer. Mice were tail bled by drawing 20  $\mu\text{L}$  freely flowing whole blood directly into an Eppendorf pipette and immediately transferring the blood into 10 mL of Cellpack diluent (TOA

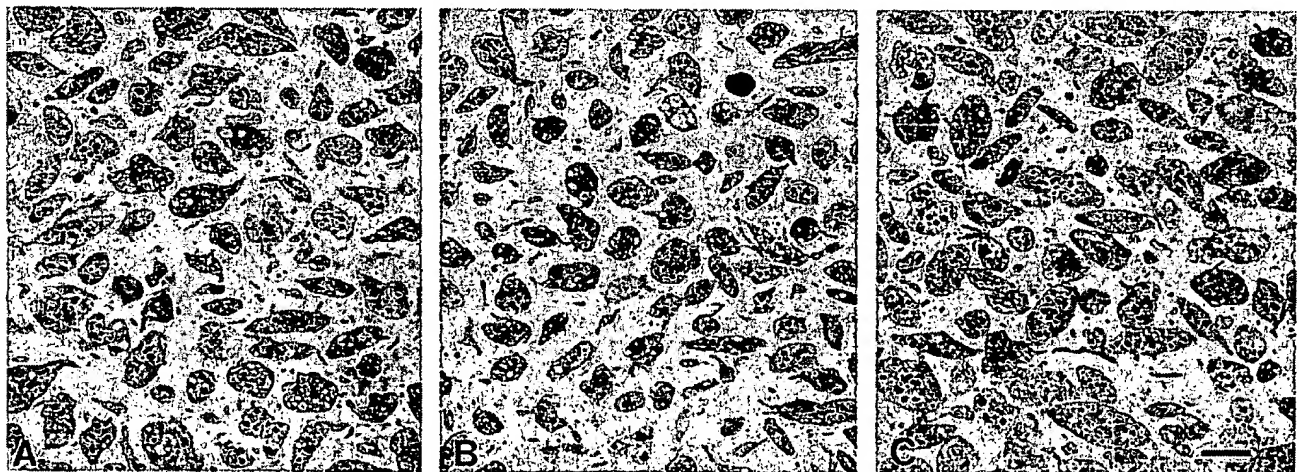


Fig 3. PEG-rHuMGDF-induced changes in MPV and PDW are demonstrated ultrastructurally in transmission electron micrographs of circulating platelets from mice treated with once daily injections of carrier (A),  $1 \mu\text{g/kg}$  (B), and  $500 \mu\text{g/kg}$  (C) of MGDF for 1 week. The platelets from mice treated with  $1 \mu\text{g/kg}$  are smaller (MPV =  $5.74 \pm 0.18$ ) and more homogeneous (PDW =  $5.40 \pm 0.28$ ) in size than those in the carrier group (MPV =  $6.14 \pm 0.11$ ,  $P < .01$ , and PDW =  $5.90 \pm 0.12$ ,  $P < .01$ ). The platelets from mice treated with  $500 \mu\text{g/kg}$  are larger (MPV =  $7.08 \pm 0.11$ ,  $P < .01$  v carrier group) and more heterogeneous in size (PDW =  $7.02 \pm 0.19$ ,  $P < .01$  v carrier group). Platelet indices were measured in individual mice and the platelets from all mice within each group ( $n = 5$  mice per group) were pooled for electron microscopy (size bar represents 2 microns).

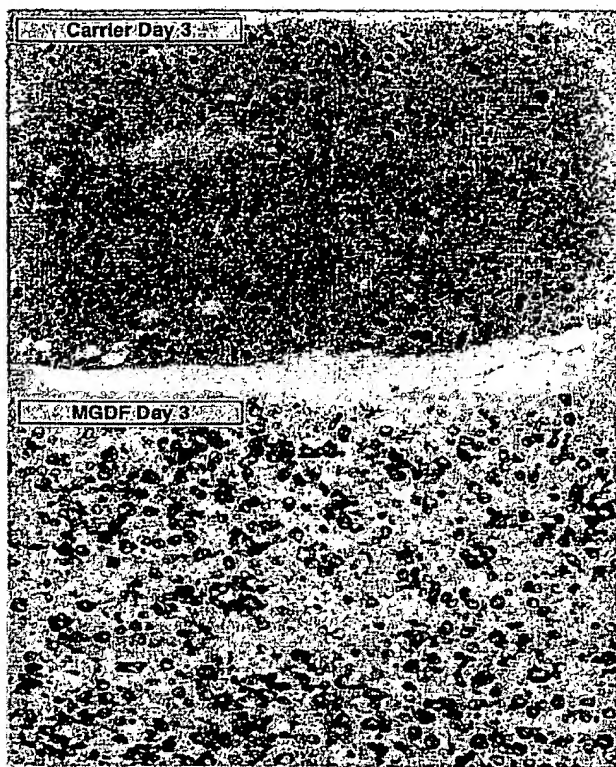


Fig 5. PEG-rHuMGDF causes a striking hyperplasia of marrow megakaryocytes at 3 days after daily injection of 500  $\mu\text{g/kg}$  MGDF as compared with carrier control (von Willebrand factor immunostain, original magnification  $\times 100$ ).

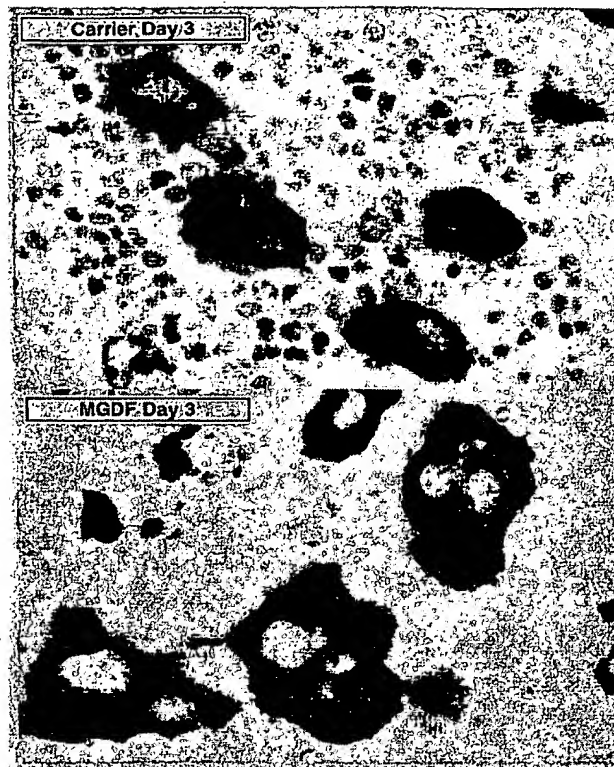
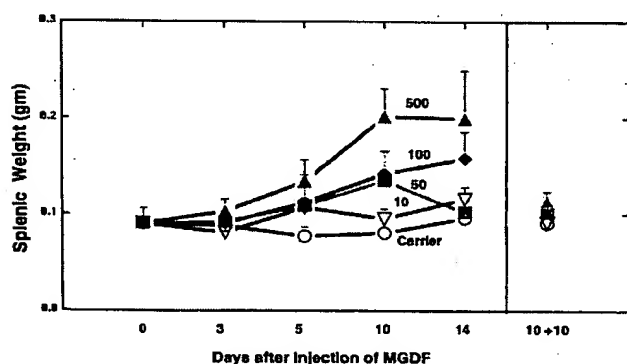


Fig 6. PEG-rHuMGDF causes an increase in the size of marrow megakaryocytes as compared with control (higher power of Fig 5, original magnification  $\times 1,000$ ).



Medical Electronics, Inc, Kobe, Japan). The blood and diluent were then inverted a few times to obtain a homogeneous mixture before counting platelets and determining platelet indices with the Sysmex cell counter within less than 20 minutes. Red and white blood cell count, platelet count, MPV, and PDW were measured with the Sysmex cell counter (Toa Medical Electronics). Carboplatin at a dose of 125 mg/kg was injected IP as a single injection on day 0 as previously described in a model of carboplatin-induced thrombocytopenia.<sup>8</sup>

Fig 7. PEG-rHuMGDF at high doses causes statistically significant splenomegaly ( $n = 10$  mice in each dosage group at each time point; results expressed as mean  $\pm$  STD). Injection of PEG-rHuMGDF for 10 days followed by cessation of treatment for 10 days results in a reversal of splenic weight to control weights ( $n = 5$  mice in each dosage group).

Fig 8. PEG-rHuMGDF injected daily at 500  $\mu\text{g/kg}$  for 10 days causes extramedullary hematopoiesis. Splenic hematopoiesis is the cause of splenomegaly with prominent increases in splenic megakaryocytes and erythropoiesis (upper left panel: original magnification  $\times 200$ ; lower left panel, original magnification  $\times 1,000$ ). Hepatic erythropoiesis (solid arrows) and myelopoiesis (arrowhead) are present (upper right panel, original magnification  $\times 1,000$ ). Megakaryocytes are present in the lung (arrows) and megakaryocyte cytoplasm is seen in the lumen of alveolar capillaries (stars) (lower right panel, original magnification  $\times 1,000$ ).

Fig 9. PEG-rHuMGDF after 10 days of daily injection at 500  $\mu\text{g/kg}$  causes not only megakaryocytic hyperplasia (original magnification  $\times 100$ , top panels), but also myeloid hyperplasia as evidenced by numerous neutrophils, erythroid hypoplasia, and lymphoid hypoplasia (original magnification  $\times 1,000$ , lower panels).

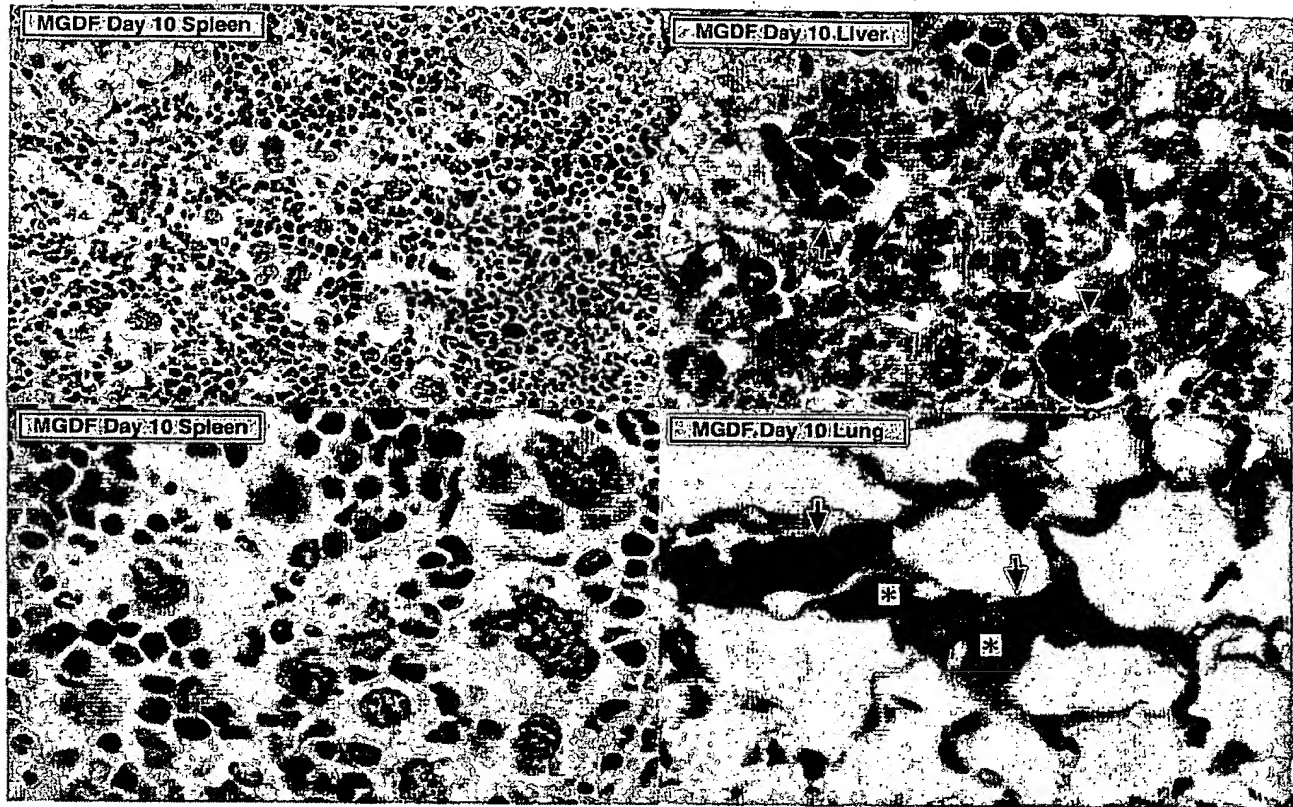


Fig 8.

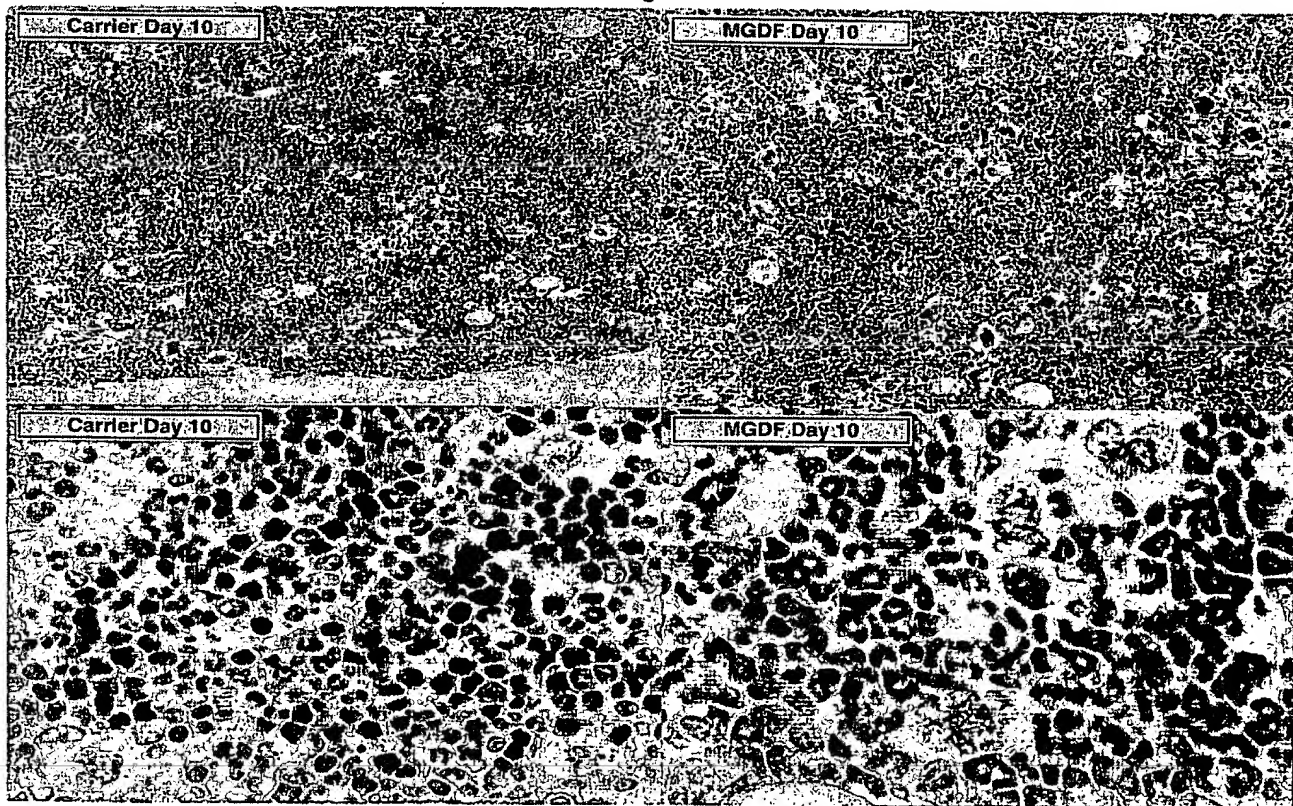


Fig 9.



Table 1. Marrow Differential on Day 10

Bone Marrow Differential (%)	Carrier (n = 5)	MGDF (500 µg/kg) (n = 5)	MGDF (10 µg/kg) (n = 5)
<b>Erythroid</b>			
Pronormoblasts	0.81 ± 0.4	0.40 ± 0.2	0.66 ± 0.21
Early normoblasts	1.15 ± 0.3	0.66 ± 0.2	1.25 ± 0.33
Intermediate normoblasts	11.56 ± 1.0	2.36 ± 0.7	8.05 ± 0.79
Late normoblasts	18.02 ± 1.0	4.94 ± 1.4	15.60 ± 1.84
<b>Myeloid</b>			
Blasts	2.96 ± 0.3	6.73 ± 1.8	3.78 ± 0.40
Promyelocytes	2.23 ± 0.7	5.65 ± 1.3	2.56 ± 0.16
Myelocytes	8.39 ± 0.9	13.62 ± 1.7	8.53 ± 0.50
Metamyelocytes	4.01 ± 0.7	7.34 ± 0.8	3.71 ± 0.60
Band cells	3.74 ± 0.3	6.46 ± 0.5	4.04 ± 0.61
Mature neutrophils	20.70 ± 1.6	31.32 ± 1.5	24.88 ± 2.17
Eosinophils	2.52 ± 0.6	3.77 ± 0.6	2.95 ± 0.78
Monocytes	2.01 ± 0.2	3.09 ± 0.6	1.84 ± 0.59
Histiocytes	1.87 ± 0.6	3.60 ± 0.5	2.16 ± 0.19
<b>Lymphoid</b>			
Lymphocytes	19.34 ± 1.2	8.62 ± 2.0	18.97 ± 3.07
Plasma cells	0.32 ± 0.2	0.27 ± 0.1	0.36 ± 0.15

The number of nucleated cells per marrow smear that were counted to obtain the marrow differentials is  $1,098 \pm 239$  cells in control smears,  $862 \pm 281$  cells in the 10 µg/kg group, and  $1,300 \pm 342$  cells in the 500 µg/kg group.

Formalin-fixed paraffin-embedded tissues were stained by hematoxylin and eosin, Masson's trichrome, or Gomori's reticulin methods. Platelet-rich fractions for electron microscopy (EM) were prepared from whole blood collected in a syringe prefilled with ACD (sodium citrate 0.085 mol/L, citric acid 0.079 mol/L, dextrose 0.180 mol/L) containing 8 µmol/L PGE<sub>1</sub>. After gentle inversions of the syringe to ensure even mixing with anticoagulant, the blood was slowly dripped into a plastic tube containing 1.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4. After fixation for 2 hours, the platelet-rich fraction was obtained by centrifugation at 400g for 20 minutes. The platelets were then pelleted at 3,200g for 10 minutes, rinsed three times in 0.1 mol/L sodium cacodylate buffer, pH 7.4, postfixed in osmium tetroxide, and embedded in LR white resin (Ted Pella, Redding, CA). Immunohistochemistry for the identification of megakaryocytes was performed with rabbit antihuman von Willebrand factor (Dako Corp, Carpinteria, CA) on 10% neutral buffered formalin-fixed, paraffin-embedded decalcified femurs sectioned longitudinally. The number of megakaryocytes in marrow and spleen per 10 randomly chosen 400× magnification fields were counted using light microscopy by an observer blinded to the experimental groups. Statistics are expressed as the mean ± 1 standard deviation, and significance was calculated by the *t*-test.

## RESULTS

PEG-rHuMGDF injected once daily IP for 2 weeks to Balb/c mice causes a dose-dependent thrombocytosis (Fig 1) with increases in circulating numbers of platelets noted at a dose as low as 1 µg/kg. A maximum increase in circulating platelets is attained at doses of 50 to 500 µg/kg (Fig 1). PEG-rHuMGDF-induced changes in MPV are also dose-related, but are more complex than the changes in circulating numbers of platelets. Although platelet numbers are increased at all doses between 1 and 500 µg/kg, the MPV can

be either decreased, normal, or increased depending on the dose and time after administration (Fig 1). Thus, PEG-rHuMGDF at a low dose (1 µg/kg) causes a decrease in MPV, PEG-rHuMGDF at an intermediate dose (10 µg/kg) causes an initial increase in MPV at day 3 followed by a decrease in MPV by day 7, and PEG-rHuMGDF at higher doses (50 to 500 µg/kg) causes an increase in MPV at days 3 to 7 followed by a gradual normalization of MPV by day 14. Interestingly, a dose-response relationship for MPV persists at doses of 50, 100, and 500 µg/kg even though platelet numbers did not increase significantly over the same dose range (Fig 1). Injection of PEG-rHuMGDF for 10 days followed by 10 days without treatment results in complete normalization of platelet numbers and MPVs to baseline levels.

PEG-rHuMGDF-induced changes in PDW, a measure of platelet size heterogeneity, parallel changes in MPV (Fig 2). A low dose (1 µg/kg) of PEG-rHuMGDF thus results in a smaller very uniform platelet population, whereas a high dose (500 µg/kg) of PEG-rHuMGDF results in a larger more heterogeneous population of platelets as compared with controls (Fig 2). PEG-rHuMGDF-induced increases and decreases in both MPV and PDW are visually recognizable in transmission electron micrographs of circulating platelets

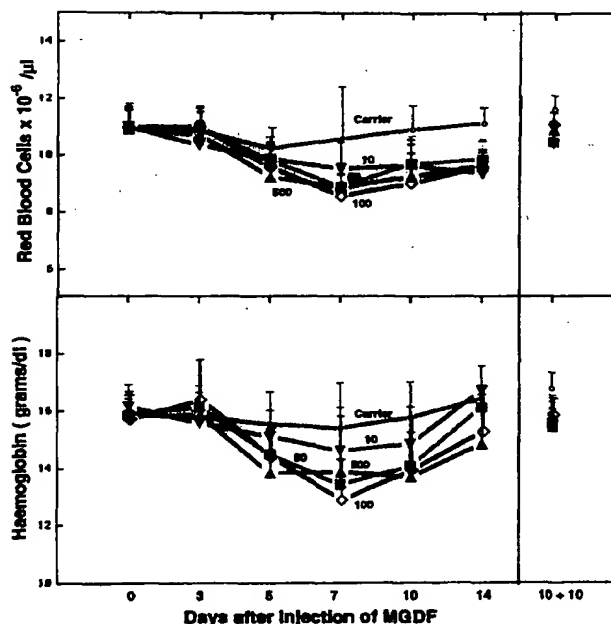
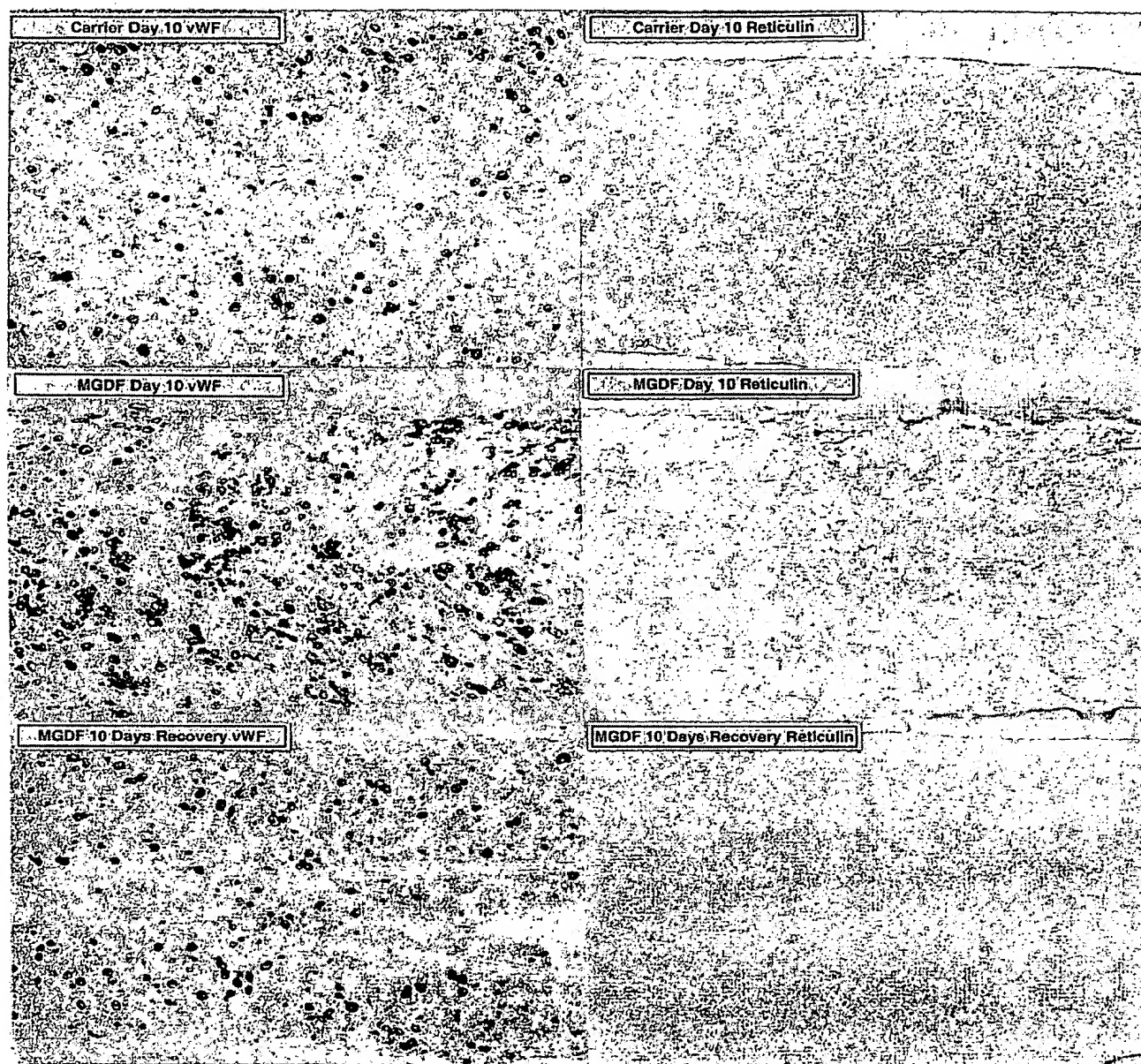


Fig 10. PEG-rHuMGDF causes a mild transient decrease in circulating numbers of red blood cells ( $P < .05$  on day 7 at PEG-rHuMGDF doses of 50 to 500 µg/kg as compared with carrier;  $P$  is not significant at 10 µg/kg,  $n = 15$  mice in each dosage group at each time point) and a mild transient decrease in hemoglobin ( $P < .01$  on day 7 at PEG-rHuMGDF doses of 50 to 500 µg/kg as compared with carrier;  $P$  is not significant at 10 µg/kg,  $n = 15$  mice in each dosage group at each time point). The decreases reach a nadir around 1 week and then begin to normalize even as daily injection of PEG-rHuMGDF continues. Injection of PEG-rHuMGDF for 10 days followed by cessation of treatment for 10 days results in normalization of red blood cell number and hemoglobin ( $n = 5$  mice in each dosage group).



**Fig 11.** PEG-rHuMGDF injected daily at 500  $\mu\text{g/kg}$  causes focal deposition of reticulin fibers in the marrow after 10 days. Reticulin fibers within the marrow parenchyma are no longer identifiable 10 days after cessation of PEG-rHuMGDF administration, demonstrating the reversibility of reticulin deposition. The number of megakaryocytes in the marrow at the corresponding timepoints is demonstrated by staining of von Willebrand factor (original magnifications  $\times 40$ ).

obtained on day 7 from mice treated with low and high doses of MGDF (Fig 3).

PEG-rHuMGDF causes an increase in marrow megakaryocytes with an increase to near peak numbers as early as 3 days after daily injections (Fig 4). The increase in both number and size of marrow megakaryocytes on day 3 is histologically dramatically demonstrable (Figs 5 and 6). Because an increase in the size of megakaryocytes may increase the number of megakaryocytes visualized per section of marrow,

the number of megakaryocytes per microscopic field is not necessarily directly proportional to the PEG-rHuMGDF-induced increase in the number of megakaryocytes per femur. In a second experiment designed to assess the appearance of megakaryocytes at 24 and 48 hours after daily injection of PEG-rHuMGDF, no significant increase in marrow megakaryocytes was noted at 24 hours, but a significant increase in marrow megakaryocytes was noted at 48 hours ( $22 \pm 1$  megakaryocytes/high-power field in controls. v  $50 \pm 3$



Fig 12. The most severe focus of PEG-rHuMGDF-induced reticulin deposition observed in the marrows of five femurs from five mice examined after 10 days of daily injection of 500  $\mu$ g/kg PEG-rHuMGDF is shown, as well as the lack of reticulin 10 days after the cessation of PEG-rHuMGDF injections (higher magnification of Fig 11, original magnification  $\times 1,000$ ).



megakaryocytes/high-power field in 100  $\mu\text{g/kg}$  PEG-rHuMGDF-treated mice,  $P < .0001$ ). A dramatic increase in marrow megakaryocytes thus occurs at 48 hours and, as might be expected, precedes the increase in circulating platelets that begins on day 3.

In contrast to marrow megakaryocytes, the number of splenic megakaryocytes is almost unchanged at day 3 and peaks at day 10 (Fig 4). The increase in splenic megakaryocytes on day 10 is accompanied by splenomegaly as manifested by splenic weight (Fig 7). Prominent extramedullary hematopoiesis, predominantly erythroid in lineage, also is noted in the spleen on day 10 (Fig 8) and contributes to the gross splenomegaly. Additional extramedullary hematopoiesis of erythroid, myeloid, and megakaryocytic lineages is noted in the liver on day 10 (Fig 8). Megakaryocytes, usually not noted in normal lungs, can easily be detected histologically in the alveolar capillary vasculature of the lung of PEG-rHuMGDF-treated mice (Fig 8). Splenomegaly (Fig 7) and extramedullary hematopoiesis are no longer evident 10 days after the cessation of 10 days of daily injection of PEG-rHuMGDF. PEG-rHuMGDF at 10  $\mu\text{g/kg}$  does not affect circulating numbers of total leukocytes, neutrophils, or lymphocytes at any time point. At the highest dose of 500  $\mu\text{g/kg}$  of PEG-rHuMGDF, a transient mild leukocytosis with an increase in neutrophils and lymphocytes is noted at 3 to 5 days after daily injection. Occasional circulating immature myeloid and erythroid cells are noted in peripheral blood smears of mice treated with 500  $\mu\text{g/kg}$ , somewhat reminiscent of a leukoerythroblastic peripheral blood reaction.

The bone marrow after 10 days of daily administration of a high dose of PEG-rHuMGDF (500  $\mu\text{g/kg}$ ) is remarkable, not only for megakaryocytic hyperplasia, but also for statistically significant myeloid hyperplasia together with erythroid and lymphoid hypoplasia (Table 1 and Fig 9). The erythroid hypoplasia in the marrow is accompanied by a mild decrease in circulating numbers of red blood cells and in hemoglobin on day 7 (Fig 10). Despite continued injection of PEG-rHuMGDF, the hemoglobin normalizes by day 14, possibly as the result of compensatory extramedullary erythropoiesis in the spleen and liver. The transient alterations in red blood cell parameters are also, of course, no longer evident 10 days after the cessation of 10 days of daily injection of PEG-rHuMGDF (Fig 10).

PEG-rHuMGDF at a high dose (500  $\mu\text{g/kg}$ ) causes the focal deposition of reticulin fibers within the marrow at 10 days after daily injection (Figs 11 and 12). A single high-power field shows the most severe reticulin deposition (Fig 12). No evidence of collagen deposition as evaluated by trichrome staining was noted at any time point after any dose of PEG-rHuMGDF. Reticulin fibers are not identifiable 10 days after the cessation of 10 days of daily injection of PEG-rHuMGDF (Figs 11 and 12).

PEG-rHuMGDF at a dose of 10  $\mu\text{g/kg}$  does not cause reticulin deposition or hematologic changes in the myeloid, erythroid or lymphoid lineages of the marrow (Table 1) and yet is effective at abrogating carboplatin-induced thrombocytopenia (Fig 13).

#### DISCUSSION

PEG-rHuMGDF is a pegylated truncated *E coli*-derived molecule related to TPO. The in vivo hematologic effects

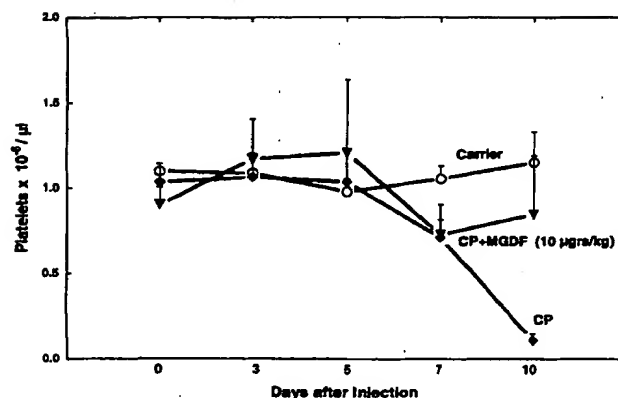


Fig 13. PEG-rHuMGDF injected IP at a dose of 10  $\mu\text{g/kg}$  beginning the day after a single IP injection of 125 mg/kg carboplatin effectively abrogates carboplatin-induced thrombocytopenia ( $n = 5$  mice at each time point; results expressed as mean  $\pm$  STD;  $P < .05$  comparing platelet numbers on day 10 in carboplatin v carboplatin plus PEG-rHuMGDF groups). PEG-rHuMGDF at 10  $\mu\text{g/kg}$  day does not cause hematologic changes in lineages other than megakaryocytes and does not cause reticulin deposition in the marrow.

of nonpegylated MGDF on platelets were previously described.<sup>8</sup> PEG-rHuMGDF is approximately 10-fold more potent than the same nonpegylated molecule as measured by its ability to increase circulating numbers of platelets in vivo in normal mice.<sup>8,9</sup> PEG-rHuMGDF is also approximately 10-fold more potent than the corresponding nonpegylated form of the molecule<sup>8,9</sup> in accelerating platelet recovery in experimental animal models of thrombocytopenia. Pegylation of MGDF, similar to pegylation of other proteins, increases its circulating half-life, which is thought to account for increased biological activity in vivo.

PEG-rHuMGDF injected daily in normal mice causes an initial increase in marrow megakaryocytes after 48 hours followed by an initial increase in circulating platelets after 72 hours. The increase in circulating platelets is accompanied by predictable, but somewhat complex, dose- and time-dependent changes in the size and size heterogeneity of platelets. In a previous study of the in vivo effect of nonpegylated *E coli*-derived MGDF on platelet number and MPV,<sup>8</sup> the MPV decreased in a simple dose-response fashion. In the present study using the more potent pegylated form, low doses also caused a uniphasic decrease in MPV. However, intermediate doses of pegylated MGDF caused an initial increase followed by a decrease in MPV, and high doses caused only an increase in MPV. The increases in circulating numbers of platelets caused by intermediate and high doses of the pegylated form are much greater than the increases caused by the nonpegylated form so that the previously reported and present results are biologically consistent. Acute stimulation of platelet production usually results in an increase in MPV.<sup>10-12</sup>

PEG-rHuMGDF-induced changes in MPV are paralleled by changes in PDW, a measure of platelet size heterogeneity whose validity was also confirmed by ultrastructural morphologic assessment of the platelets. The cellular mechanism

that gives rise to the observed decreases and increases in MPV and PDW is unknown. One possible hypothesis is that PEG-rHuMGDF accelerates the formation of the intracellular demarcation membranes within megakaryocytes. A mild stimulation of demarcation membrane formation by low doses of PEG-rHuMGDF might result in more, but smaller, platelets per megakaryocyte. Pharmacologically extremely high doses of PEG-rHuMGDF might result in discordant generation of megakaryocyte mass and demarcation membranes resulting in the shedding of larger fragments of megakaryocyte cytoplasm consistent with the observed formation of megathrombocytes and "stress" platelets, as well as increased MPV and PDW.

At suprapharmacologic doses of PEG-rHuMGDF, the hyperplasia of megakaryocytes in the marrow becomes so extreme that erythroid and lymphoid hypoplasia occur. Similar marrow hypoplasia of the erythroid and lymphoid lineages has been previously described in rodents after daily administration of large doses of other growth factors.<sup>13-15</sup> The bone marrow erythroid hypoplasia is accompanied by the development of splenic and hepatic extramedullary myelopoiesis with prominent erythropoiesis. Extramedullary hematopoiesis is also commonly observed in rodents following the administration of high doses of recombinant hematologic growth factors. The mechanism of myeloid marrow hyperplasia in the face of erythroid hypoplasia is unknown. The presence within the pulmonary circulation of megakaryocytes whose substantial cytoplasm is distorted within the confines of the narrow alveolar capillary network is of interest in regards to the hypothesis that platelets are produced in the pulmonary circulation by a physical process.<sup>16</sup>

Deposition of reticulin fibers in the marrow was noted after injection of high doses of PEG-rHuMGDF, possibly related to a local increase in the concentration of platelet-associated mediators of extracellular matrix deposition such as PDGF. Of note is the complete and rapid reversibility of reticulin deposition after cessation of administration of PEG-rHuMGDF, demonstrating that in addition to the responsiveness of the marrow's hemopoietic cellular elements to growth factors, the structural support stroma of the marrow demonstrates surprising plasticity. In this regard, it is of interest to note that secondary myelofibrosis in cancer patients has also been noted to be reversible.<sup>17</sup>

Patients with idiopathic myelofibrosis may present with many hematopathologic findings including anemia, increased numbers of marrow megakaryocytes, myeloid hyperplasia, erythroid and lymphoid hypoplasia, deposition of marrow reticulin, splenomegaly, and extramedullary hematopoiesis.<sup>18-19</sup> The extent of pathologic deposition of marrow connective tissue in idiopathic myelofibrosis varies from focal increased reticulin deposition to diffuse collagen deposition and osteosclerosis. Marrow collagen deposition and osteosclerosis were not observed in the mice in the present study, although Yan et al<sup>20</sup> found osteosclerosis in mice that chronically and constitutively overexpress very high levels of circulating thrombopoietin as the result of retroviral thrombopoietin gene transfer using bone marrow cells to lethally irradiated mice. The constellation of pathologic features caused by suprapharmacologic doses of PEG-

rHuMGDF supports the hypothesis of previous investigators<sup>21-24</sup> that some of the clinical features of some cases of myelofibrosis and acute myelofibrosis may represent a paraneoplastic syndrome caused by a neoplastic proliferation of cells of megakaryocytic phenotype. Finally, PEG-rHuMGDF's ability to ameliorate thrombocytopenia in experimental animal models at doses that do not cause undesirable hematologic effects supports the promise of PEG-rHuMGDF as a clinically valuable therapeutic.

## REFERENCES

1. Nichol JL, Hornkohl AC, Choi ES, Hokom MM, Ponting I, Scheunig FW, Hunt P: Enrichment and characterization of peripheral blood-derived megakaryocyte progenitors that mature in short term liquid culture. *Stem Cells* 12:494, 1994
2. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, Chang M-S, Samal B, Nichol JL, Swift S, Johnson MJ, Hsu R-Y, Parker VP, Suggs S, Skrine JD, Merewether LA, Clogston C, Hsu E, Hokom M, Hornkohl A, Choi E, Pangelinan M, Sun Y, Mar V, McNinch J, Simonet L, Jacobsen F, Xie C, Shutter J, Chute H, Basu R, Selander L, Trollinger D, Sieu L, Padilla D, Trail G, Elliott G, Izumi R, Covey T, Crouse J, Garcia A, Xu W, del Castillo J, Biron J, Cole S, Hu MC, Pacifici R, Ponting I, Saris C, Wen D, Yung YP, Lin H, Bosselman RA: Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 77:1117, 1994
3. Lok S, Kaushansky K, Holly R, Kuijper JL, Lofton-Day CE, Oort PJ, Grant FJ, Heipel MD, Burkhead SK, Kramer JM, Bell LA, Sprecher CA, Blumberg H, Johnson R, Prunkard D, Ching AFT, Mathewes SL, Bailey M, Forstrom JW, Buddle MM, Osborn SG, Evans SJ, Sheppard PO, Presnell SR, O'Hara PJ, Hagen FS, Roth GJ, Foster DC: Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* 369:565, 1994
4. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, Methia N, Breton-Gorius J, Cosman D, Vainchenker W: c-Mpl ligand is a humoral regulator of megakaryocytopoiesis. *Nature* 369:571, 1994
5. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, et al: Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* 369:533, 1994
6. Kuter DJ, Beeler DL, Rosenberg RD: The purification of megakaryopoietin: A physiologic regulator of megakaryocyte growth and platelet production. *Proc Natl Acad Sci USA* 91:11104, 1994
7. Kato T, Ogami K, Shimada Y, Iwamatsu A, Sohma Y, Akahori H, Horie K, Kokubo A, Kudo Y, Maeda E, Kobayashi K, Ohashi H, Ozawa T, Inoue H, Kawamura K, Miyazaki H: Purification and characterization of thrombopoietin. *J Biochem* 118:229, 1995
8. Ulich TR, del Castillo J, Yin S, Swift S, Padilla D, Senaldi G, Bennett L, Shutter J, Bogenberger J, Sun D, Samal B, Shimamoto G, Lee R, Steinbrink R, Boone T, Sheridan WT, Hunt P: Megakaryocyte growth and development factor ameliorates carboplatin-induced thrombocytopenia in mice. *Blood* 86:971, 1995
9. Hokom MM, Lacey D, Kinsler O, Choi E, Kaufman S, Faust J, Rowen C, Dywer E, Nichol JL, Grasel T, Wilson J, Steinbrink R, Hecht R, Grampp G, Bartley T, Li Y-S, Boone T, Hunt P: Pegylated megakaryocyte growth and development factor abrogates the lethal thrombocytopenia associated with carboplatin and irradiation in mice. *Blood* 86:4486, 1995
10. Corash L, Chen HY, Levin J, Baker G, Lu H, Mok Y: Regulation of thrombopoiesis: Effects of the degree of thrombocytopenia on megakaryocyte ploidy and platelet volume. *Blood* 70:177, 1987
11. Corash L, Mok Y, Levin J, and Baker G: Regulation of plate-

let heterogeneity: Effects of thrombocytopenia on platelet volume and density. *Exp Hematol* 18:205, 1990

12. Corash L: The relationship between megakaryocyte ploidy and platelet volume. *Blood Cells* 15:81, 1989

13. Ulich TR, del Castillo J, McNiece I, Yi E, Alzona C, Yin S, Zsebo C: Stem cell factor in combination with G-CSF or GM-CSF synergistically increases granulopoiesis in vivo. *Blood* 78:1954, 1991

14. Ulich TR, del Castillo J, Yin S: TNF exerts dose-dependent effects on erythropoiesis and myelopoiesis in vivo. *Exp Hematol* 18:311, 1990

15. Ulich TR, del Castillo J, Yi ES, Yin S, McNiece I, Yung YP, Zsebo KM: Hematologic effects of stem cell factor in vivo and in vitro in rodents. *Blood* 78:645, 1991

16. Martin JF, Slater DN, Trowbridge EA: Evidence that platelets are produced in the pulmonary circulation by a physical process, in Levine RF, Williams N, Levin J, Evatt BL (eds): *Megakaryocyte Development and Function*. New York, NY, Liss, 1986, p 405

17. Athens JW: Myelofibrosis, in Lee GR, Bithell TC, Foerster J, Athens JW, Lukens JN (eds): *Wintrobe's Clinical Hematology* (ed 9). Philadelphia, PA, Lea & Febiger, 1993, p 2030

18. Buhr T, Choritz H, Georgii A: The impact of megakaryocyte proliferation for the evolution of myelofibrosis: Histologic follow-

up study in 186 patients with chronic myeloid leukemia. *Virchows Arch A Pathol Anat Histopathol* 420:473, 1992

19. Thiele J, Windecker R, Kvasnicka H, Titius B, Zankovich R, Fischer R: Erythropoiesis in primary idiopathic osteomyelofibrosis. *Am J Hematol* 46:36, 1994

20. Yan XQ, Lacey D, Fletcher F, Hartley C, McElroy T, Sun Y, Xia M, Mu S, Saris C, Hill D, Hawley RG, McNiece I: Chronic exposure to retroviral vector encoded MGDF (mpl ligand) induced lineage specific growth and differentiation of megakaryocytes in mice. *Blood* 86:4025, 1995

21. Castro-Malaspina H: Pathogenesis of myelofibrosis: Role of ineffective megakaryopoiesis and megakaryocyte components, in *Myelofibrosis and the Biology of Connective Tissue Disease*. New York, NY, Liss, 1984, p 427

22. Castro-Malaspina, Moore MAS: Pathophysiologic mechanisms acting in the development of myelofibrosis: Role of megakaryocytes. *Nouv Rev Fr Hematol* 24:221, 1982

23. Castro-Malaspina H, Rabellino E, Yen A, Nachmann RL, Moore MAS: Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. *Blood* 57:781, 1981

24. Bain B, Catovsky D, O'Brien M, Prentice G, Lawlor E, Kumar T, McCann S, Matutes E, Galton D: Megakaryoblastic leukemia presenting as acute myelofibrosis. *Blood* 58:206, 1981